A STUDY OF THE PATHOLOGICAL LESIONS IN RATS EXPOSED TO PAVETAMINE

5.1 INTRODUCTION

The active principle of plants inducing gousiekte was isolated in 1995 and identified as pavetamine (Fourie *et al.* 1995; R Vleggaar, unpublished data 1997). The following criteria were adhered to during the isolation process to ensure that gousiekte was induced:

- The animal models used were sheep or goats since, at the time, no evidence could be found that laboratory animals were susceptible to the intoxication.
- Cardiac failure had to follow after an appropriate latent period and the disease had to be confirmed histopathologically.

Subsequent studies in rats have confirmed the cardiotoxicity of extracts from *Pavetta harborii* in this species (Pipedi, 1999; Hay *et al.* 2001; Schultz *et al,* 2001). Limited information is available on the myocardial lesions in rats since previous work was based on studies using plant extracts, and tissues were not examined light-microscopically (Hay *et al.* 2001).

The primary objective of this study was to document and compare the myocardial lesions in rats exposed to pavetamine with lesions reported in rats exposed to plant extracts from *Pavetta harborii.* A secondary objective was to compare the lesions induced in rats with lesions recorded in sheep exposed to dried *Pachystigma pygmaeum* and *Fadogia homblei* plant material, as outlined in chapters 3 and 4.

5.2 MATERIALS AND METHODS

5.2.1 Pavetamine extraction

Pavetamine was extracted from *P. harborii* as outlined by Fourie *et al.* (1995) and freeze dried. The toxin was dissolved in normal saline at a concentration of 2 mg/ml immediately before administration. Control rats received normal saline.

5.2.2 Experimental animals and dosing regimen

The investigation complied with the *Guide for the care and use of laboratory animals* (National Institute of Health 1996). Ethics approval was obtained from the ARC-OVI Animal Ethics Committee.

To confirm the toxicity and determine the dosage rate of the batch of pavetamine used in the experiment an adult Sprague-Dawley rat was injected intraperitoneally (i.p.) with pavetamine at a dosage rate of 10 mg/kg on day 0. The dosage rate was based on results of previous trials using pavetamine prepared in the same way. The animal showed clinical signs of lethargy and inappetence within 48 hours and was euthanased by an i.p. administration of an overdose of pentobarbitone sodium (Euthapent, Kyron Laboratories (Pty) Ltd.).

In the main experiment, 14 healthy, young, male Sprague-Dawley rats of the same age were used and divided equally into a control group and a treated group. Individuals were kept in separate cages. The animals had free access to water and nutritionally balanced rat cubes (Epol (Pty) Ltd SA). Each animal was weighed twice a week using a top pan balance (Shimadzu Libror, Model EB-4000, capacity 4 000 g, readability 0,01 g) and observed daily for signs of a ruffled coat and any behavioural abnormality such as lethargy.

Seven rats were injected i.p. with pavetamine at a dosage rate of 5 mg/kg on day 0. Three were killed on day 6 with an overdose of pentobarbitone sodium and the remaining four were injected with pavetamine at a dosage rate of 3 mg/kg on day 27 and euthanased on day 42. Control rats were injected i.p.

with normal saline on the same days. This information is summarised in table 5.1.

TABLE 5.1 Rats exposed to pavetamine: Dosing regimen, fate and light-microscopical myocardial lesions

ANIMAL NUMBER	DOSING REGIMEN AND FATE	LIGHT-MICROSCOPICAL LESIONS
P1	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0. Euthanased on day 6	No lesions
P2	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0. Euthanased on day 6	No lesions
P3	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0. Euthanased on day 6	No lesions
P4	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0 and 3 mg/kg on day 27. Euthanased on day 42	Mild multifocal myocardial necrosis with fibrosis and round cell infiltration
P5	Injected with pavetamine at a dosage rate of 5mg/kg on day 0 and 3mg/kg on day 27. Euthanased on day 42	Moderate multifocal myofibre necrosis with round cell infiltration
P6	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0 and 3 mg/kg on day 27. Euthanased on day 42	Mild multifocal myocardial necrosis with fibrosis and round cell infiltration
P7	Injected with pavetamine at a dosage rate of 5 mg/kg on day 0 and 3 mg/kg on day 27. Euthanased on day 42	Mild multifocal myocardial necrosis with fibrosis and round cell infiltration

5.2.3 Pathology

5.2.3.1 Transmission electron microscopy (TEM)

Sample collection of the experimental animals commenced immediately after the animals had been euthanased.

A transmural longitudinal section of the middle of the left free ventricular wall, extending from the left atrium to the apex and approximately 1 mm in thickness, was collected and divided in half to separate the subepicardial tissue from the subendocardial tissue. Randomly selected cubes measuring 0,5 mm^3 to 1 mm^3 were cut from each half and fixed in 2,5 % glutaraldehyde (pH 7,2 to pH 7,4) for 24 hours. Selected blocks were post-fixed in 2 % osmium tetroxide for one hour, dehydrated in a graded ethanol series (50–100 %), passed through propylene oxide as the intermediate solvent, and embedded in EM Bed 812. Thick (1–2 µm) sections were cut for tissue orientation and stained with toluidine blue. Thin sections from selected blocks were stained at room temperature for 20 minutes in a saturated aqueous solution of uranyl acetate, rinsed and then post-stained for three minutes in Reynold's lead citrate.

5.2.3.2 Light-microscopy

Animals were necropsied after specimens for TEM had been collected. After a complete necropsy, samples were taken from various organs from each case, including the heart, lungs, liver, spleen, kidney, gastrointestinal tract, skeletal muscles and brain, and immersed in 10 % buffered formalin. The samples were routinely processed for histopathological examination and stained with haematoxylin and eosin (HE). Two transmural planes were sectioned from the left free ventricular wall to allow examination of both the endo- and epicardium. Selected sections were stained with Masson's trichrome stain for collagen (Armed Forces Institute of Pathology 1968).

5.2.3.3 Imaging analysis

For imaging analysis, sections from randomly selected blocks processed for TEM (*vide supra)* from both the subepicardial and subendocardial tissue from four control animals euthanased on day 42 (control group) and the four rats

exposed to pavetamine on day 0 and day 27 (affected group) were photographed with a Philips CM10 TEM operated at 80 KV. The scanned photomicrographs were imported to a drawing template of the 1TEM software imaging system. The photomicrographs were scaled to the original print of the photograph by using the "bar". Measurements were taken with the 1TEM soft imaging system.

From each animal the following measurements were taken of nine to thirteen randomly selected fibres with full nuclear profiles in the subendocardial and subepicardial tissue: myofibre diameter at the level of the centre of the nucleus, nucleus perimeter, and nucleus area.

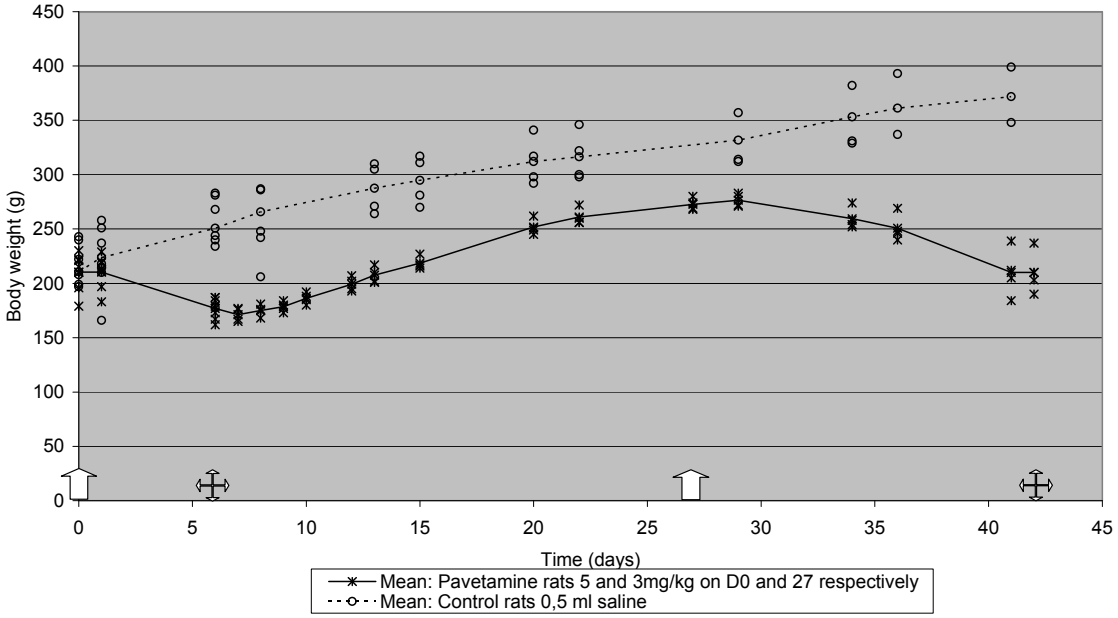
5.2.3.4 Statistical analysis

Within each of the affected and control groups, mean fibre diameter, nucleus perimeter and nucleus area were compared between the epi- and endocardium using analysis of variance, blocking on animal, i.e. with each animal serving as its own control. The analysis was done using Stata 10.1 (StataCorp., College Station, Texas, USA).

5.3 RESULTS

5.3.1 Clinical signs

Rats exposed to pavetamine had a slower weight gain than the controls, became lethargic with signs of inappetence between day 3 and day 8 after exposure and regained weight from about day 10 to day 30. This is illustrated in figure 5.1 and table 5.2. After the second administration of pavetamine on day 27 the treated group showed a further progressive weight loss that persisted until the end of the experiment. The animals had a ruffled coat from day 36 onwards and became increasingly weak towards the end of the experiment. For ethical reasons, it was decided to terminate the experiment on day 42.



Key

⇧ i.p. administration

✥ euthanased

Figure 5.1 Body weight (g) gain of control rats and rats exposed to pavetamine on day 0 and day 27

TABLE 5.2 Effect of pavetamine on body weight (g) of rats

DAY	GROUP	RATS							MEAN WEIGHT (g)
		1	2	3	4	5	6	7	
0	Pavetamine	230	222	215	210	179	221	196	210,4
	Control	166	208	222	243	240	197	199	210,7
6	Pavetamine	187	179	167					177,7
	Control	206	244	268					239,3
41	Pavetamine				239	205	184	212	210,0
	Control				399	393	347	348	371,8

5.3.2 Macroscopical examination

Apart from macroscopical changes associated with weight loss, for example generalised atrophy of skeletal muscles, no lesions were evident in any of the rats exposed to pavetamine.

5.3.3 Light-microscopical examination

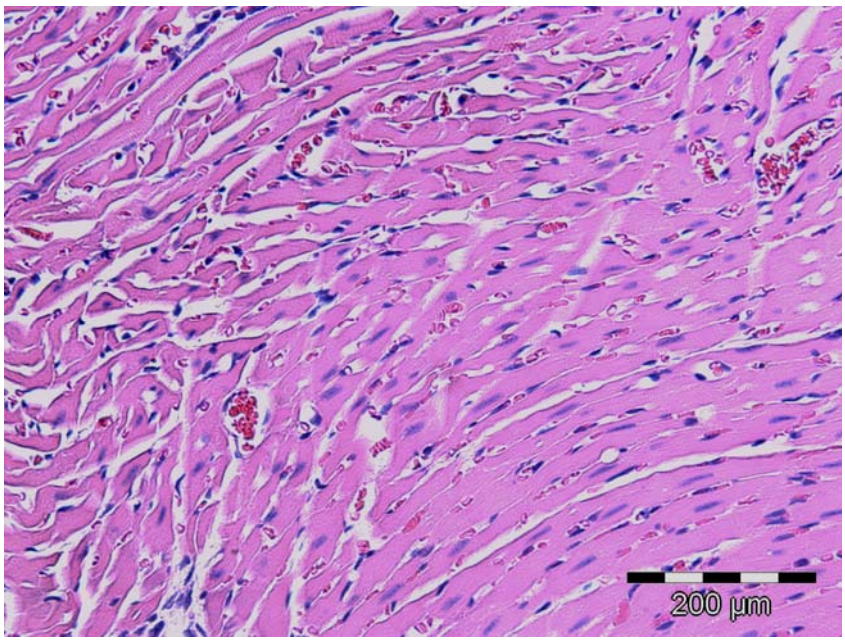


Figure 5.2 Normal myocardium of a control rat. HE

Normal myocardium of a control rat is depicted in figure 5.2 and the light-microscopical lesions are summarised in table 5.1. In both control and experimental animals variation in the diameter of the myofibres was conspicuous throughout the myocardial wall, which made it impossible to identify atrophic fibres on the basis of light-microscopical observations alone. No lesions were noted in the three experimental rats euthanased on day 6.

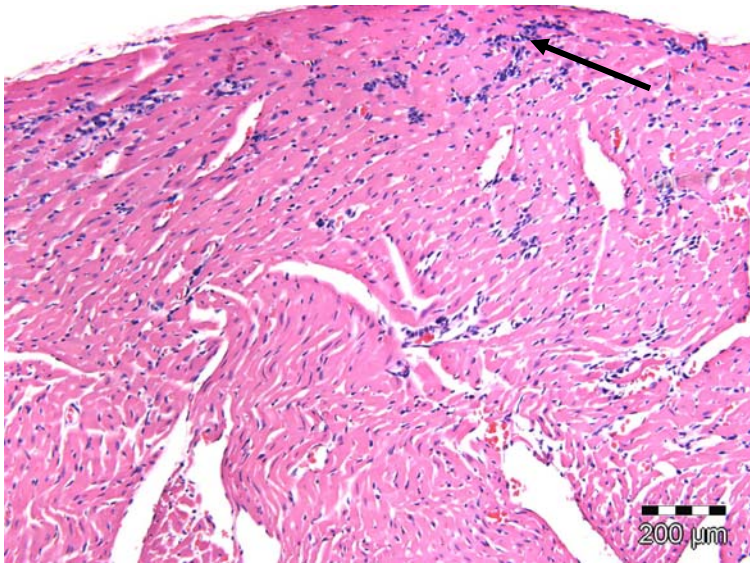


Figure 5.3 Multifocal round cell infiltration in the myocardium (arrow) of rat P4 exposed to pavetamine and euthanased on day 42. HE

In the four pavetamine-treated rats euthanased on day 42 lesions were present throughout the myocardial wall and comprised multifocal necrosis of single or small groups of myofibres with an associated replacement fibrosis and mild to moderate round cell infiltration (figs 5.3, 5.4, 5.5). The extent of the lesions ranged from mild in rats P4, P6 and P7 to moderate in rat P5. Masson's trichrome stain was beneficial in identifying the replacement fibrosis associated with the myofibre necrosis. Myofibres in close proximity to the necrotic foci appeared swollen with a loss in striation. No lesions were present in any of the other organs examined.

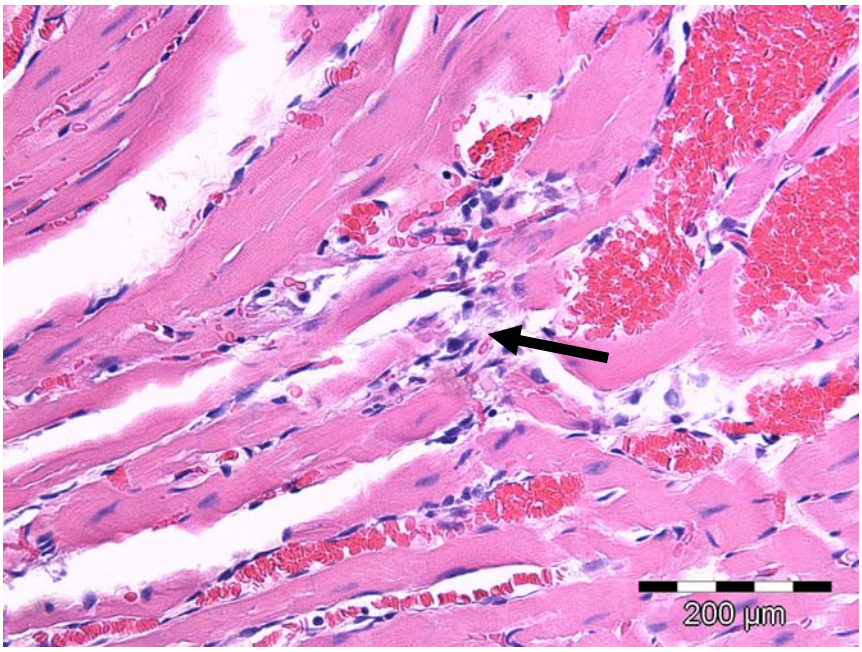


Figure 5.4 Focal myocardial necrosis with an associated round cell infiltration (arrow) in rat P5 exposed to pavetamine. HE

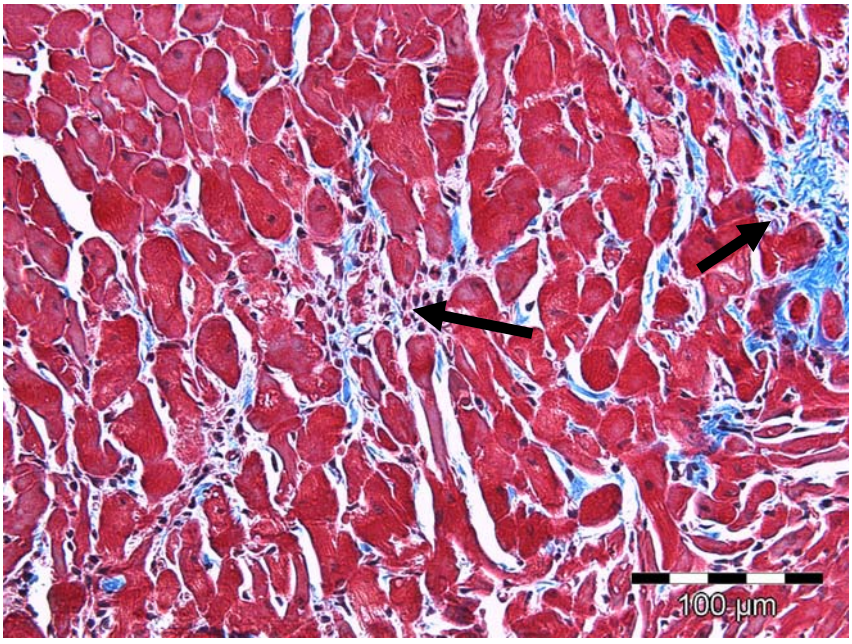


Figure 5.5 Replacement fibrosis associated with myofibre necrosis (arrows) in rat P5 injected with pavetamine and euthanased on day 42

5.3.4 Transmission electron microscopical examination

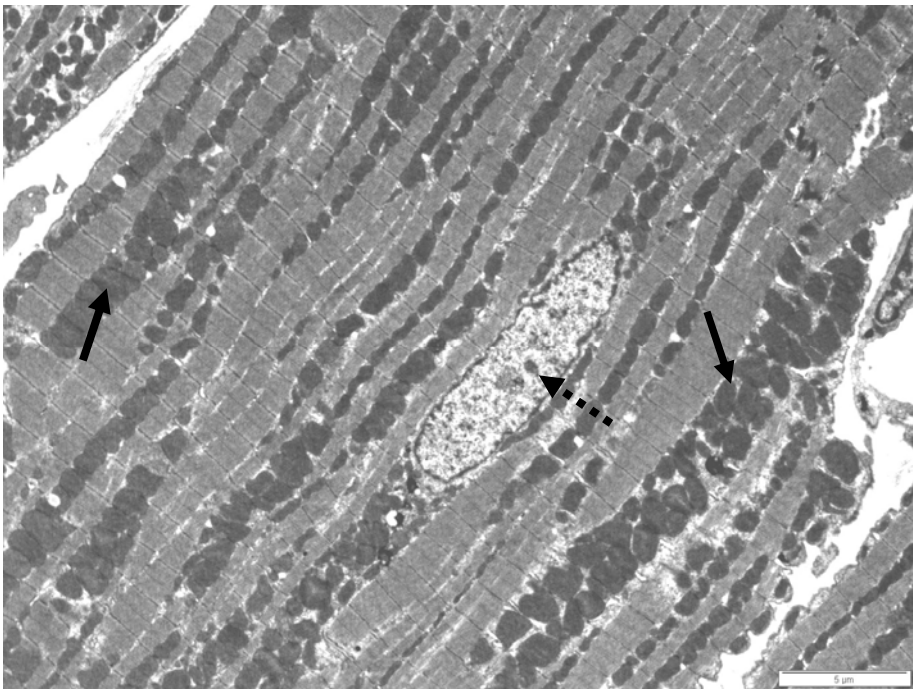


Figure 5.6 Myofibre of a control rat with a nucleus (dotted arrow) in the centre. Note the distribution of the mitochondria (solid arrows) and myofibril diameter

Myofibre of a control rat is depicted in figure 5.6. Degenerative myofibres in rats euthanased on day 42 revealed nuclear changes associated with necrosis, including changes in the distribution of chromatin, for example karyolysis characterised by an intact nuclear envelope with a partial lost of nuclear contents (fig 5.7). Also present was swelling of the mitochondria with a loss of normal morphology owing to ballooning of the cristae (fig 5.8). Swollen mitochondria contained intramitochondrial dense matrical deposits (flocculent or woolly densities). Some of these deposits were very electron dense and the woolly nature was hard to discern. The dense matrical deposits represent an early manifestation of irreversible cell injury.

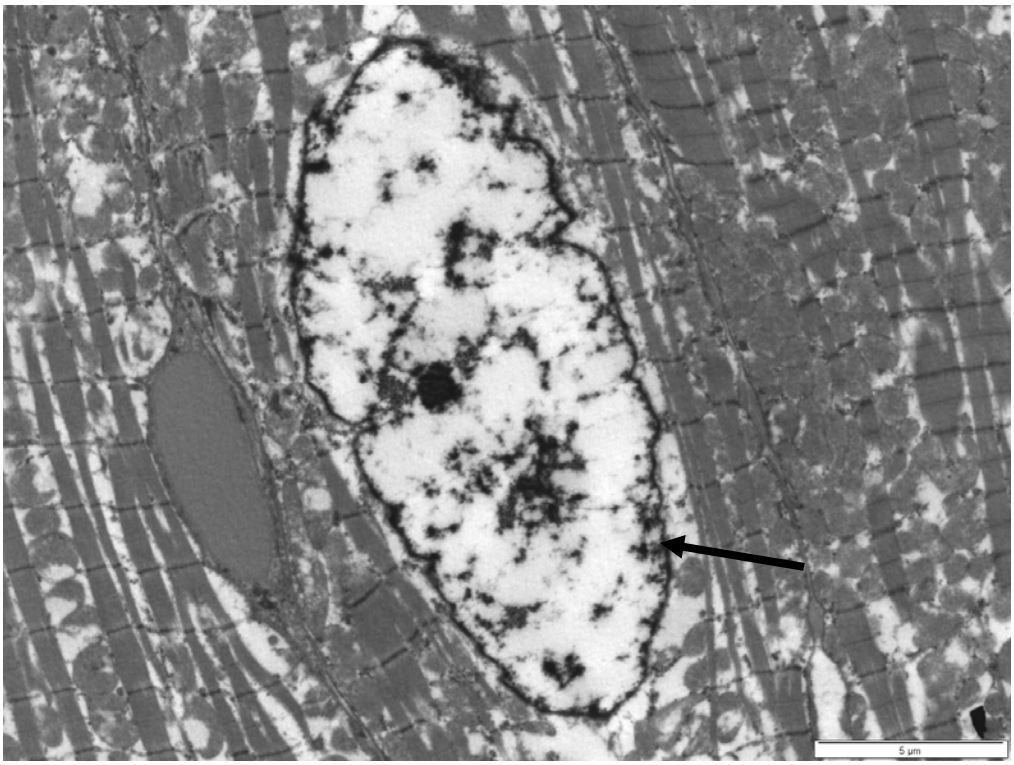


Figure 5.7 Myofibre with necrotic nucleus evidenced by karyolysis. The nuclear envelope is still intact (arrow)

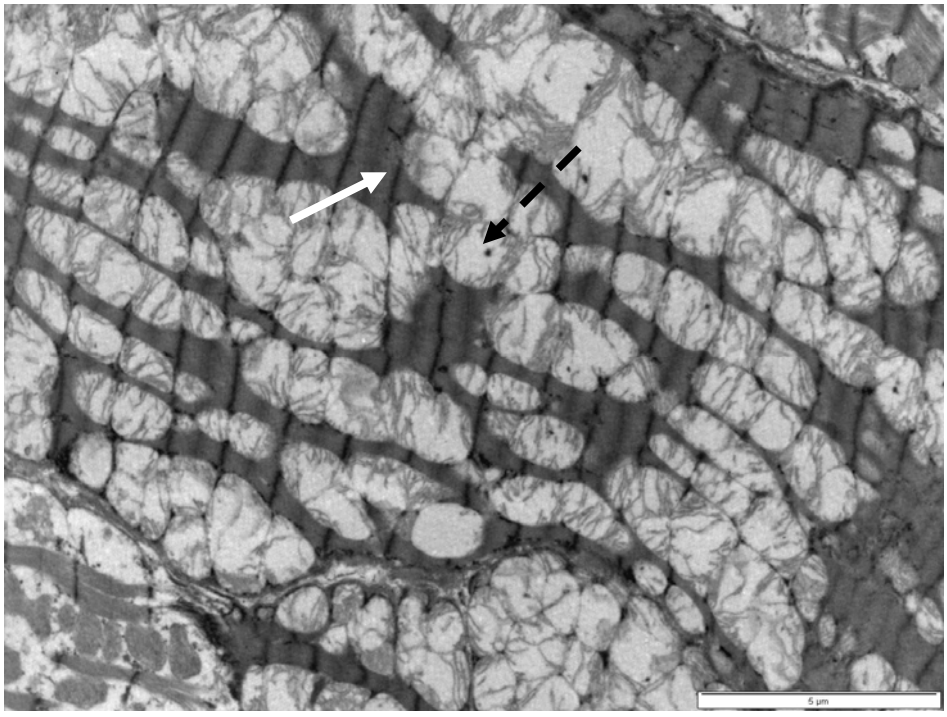


Figure 5.8 Swelling of mitochondria (white arrow) with the presence of dense matrical deposits (dashed arrow)

In rat P5, the animal with the more severe light-microscopical lesions, the diameter of some myofibrils varied significantly compared to the control rats and large, empty inter-fibrillar spaces could be discerned (fig. 5.9). Also present in the affected fibres were scattered areas of early myofibrillar loss affecting one or more adjacent sarcomeres (fig. 5.10). Separation of the opposing membranes at the level of the intercalated discs was a regular observation in the treated animals but was also present in one of the controls.

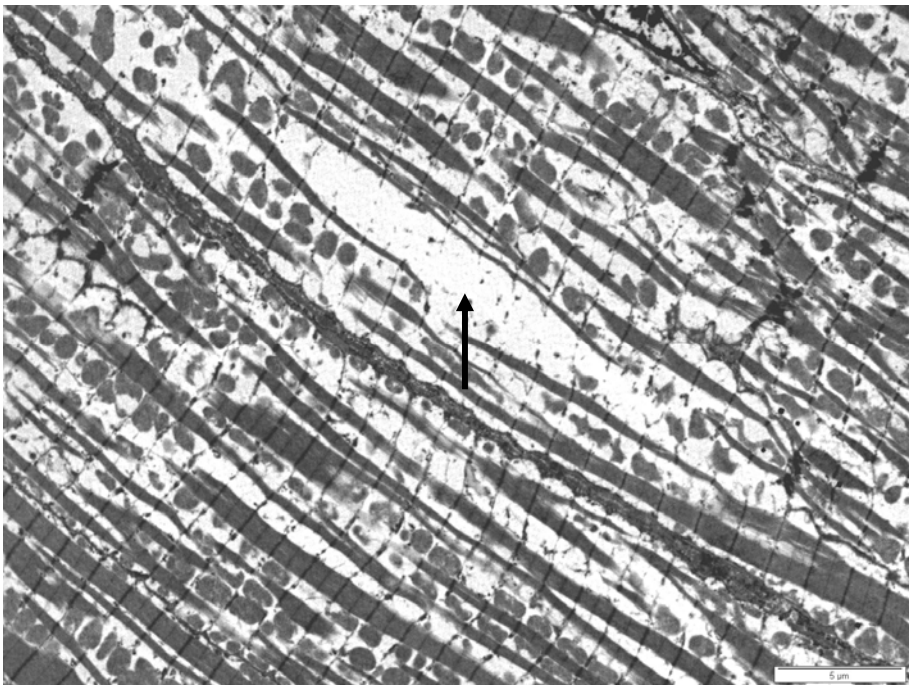


Figure 5.9 Myofibre of rat P5 euthanased on day 42. Note the variation in myofibril diameters with loss of contact between myofibrils (arrow)

5.3.5 Statistical analysis

The mean myofibre diameter, nucleus perimeter and nucleus area in the endo- and epicardial regions of the control and affected groups are outlined in tables 5.3, 5.4, 5.5 and 5.6.

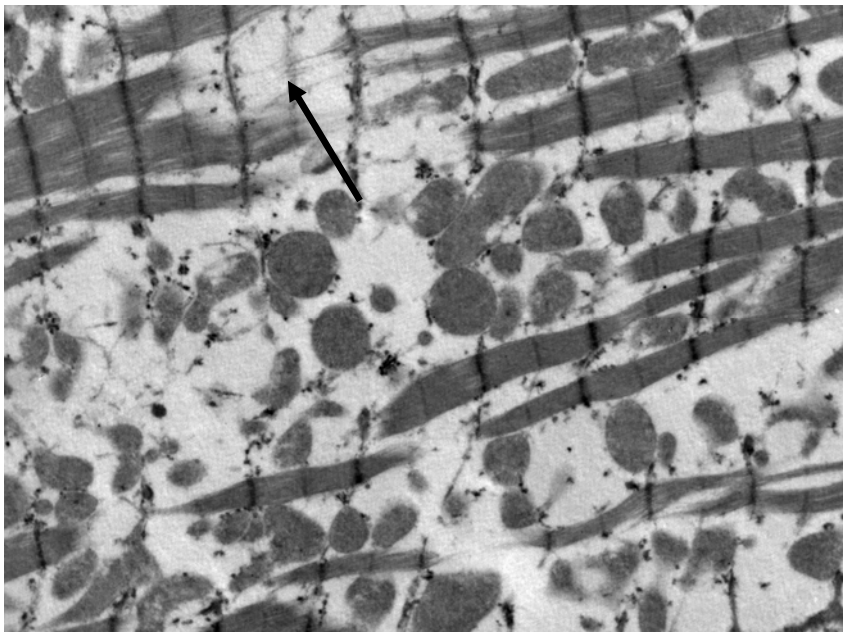


Figure 5.10 Myofibre of a rat (P5) exposed to pavetamine and euthanased on day 42. Note the segmental myofibrillar lysis (arrow)

Table 5.3 Measurements in the endocardial region of the control group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter (µm)	37	24,16	4,85	14,88	36,53
Nucleus perimeter (µm)	37	36,21	7,14	25,12	52,28
Nucleus area (μm^2)	37	40,36	15,55	19,28	77,27

Table 5.4 Measurements in the epicardial region of the control group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter(µm)	42	21,49	5,44	13,27	38,09
Nucleus perimeter (µm)	41	31,20	6,48	18,23	41,87
Nucleus area (μm^2)	41	32,42	11,32	16,89	58,51

Table 5.5 Measurements in the endocardial region of the affected group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter (µm)	50	16,64	4,83	6,79	32,05
Nucleus perimeter (µm)	50	36,22	8,72	17,68	52,32
Nucleus area (μm^2)	50	41,14	21,10	13,70	122,15

Table 5.6 Measurements in the epicardial region of the affected group

Variable	Observations	Mean	Standard deviation	Minimum	Maximum
Myofibre diameter(µm)	45	17,58	4,36	10,53	29,11
Nucleus perimeter (µm)	45	36,10	8,03	23,49	62,51
Nucleus area (μm^2)	45	39,62	17,48	16,04	108,96

The results of the analysis of variance (ANOVA) showed that the mean myofibre diameter, nucleus perimeter and nucleus area in the control group were significantly greater in the subendocardial tissue than in the subepicardial tissue (P = 0,022, P = 0,002 and P = 0,009, respectively) (tables 5.3, 5.4). On the other hand, the mean myofibre diameter, nucleus perimeter and nucleus area in the affected group revealed no statistically significant difference between subendocardial and subepicardial tissues (P = 0,533, P = 0,890 and P = 0,840, respectively) (tables 5.5, 5.6). This suggests that in the affected group the decrease in fibre measurements were more substantial in the subendocardial fibres than in the subepicardial fibres, which was taken as evidence of atrophy.

5.4 DISCUSSION

This study corroborated the reports of cardiotoxicity in rats reported by previous workers (Hay *et al.* 2001; Hay, Schultz & Schutte 2008). Multifocal myocardial necrosis throughout the myocardial wall, with an associated round cell infiltration and replacement fibrosis, was the most striking light-microscopical lesion in rats that had been dosed twice (on day 0 and day 27) and euthanased on day 42. Ultrastructural lesions in degenerative/necrotic fibres included karyolysis, swelling of the mitochondria with the presence of dense matrical deposits, focal lysis of myofilaments, and a remarkable variation in the diameter of myofibrils within the same myofibre with loss of contact between myofibrils in one rat.

As a general rule the myocardial lesions were mild. In both the control rats and those exposed to pavetamine there was a noticeable variation in the myofibre diameter, which made it extremely difficult to identify atrophic fibres on the basis of histopathological examination alone. Although there is a general notion that myocardial myocytes are terminally differentiated, approximately 15 % to 20 % of myocytes in rats retain the capacity to replicate (Kajstura *et al.* 2000). Furthermore, those that retain the capacity to replicate change with age so that there is no point during the life span at which all myocytes are comparable in

terms of age, size, shape and molecular properties. This may explain the variation in size noted in the myocytes.

In one of the experimental animals euthanased on day 42 (P5) myofibre atrophy, characterised by remarkable variation in myofibrillar diameter, loss of contact between myofibrils, and segmental myolysis of myofibrils, was observed with electron microscopy. The lesions resembled the changes reported in sheep with a short latent period as outlined in chapter 3. The mitochondrial proliferation reported in the hearts of rats exposed to *P. harborii* extract for three successive days and euthanased three weeks after the first administration by Hay *et al.* (2001) was not observed in the current study. Furthermore, folding of the nuclear membrane reported by these authors was seen in both treated and control animals in the current study and was often associated with the presence of contraction bands. In the current study the absence of lesions in rats exposed to pavetamine once and euthanased on day 6 was most probably due either to insufficient time for lesions to develop owing to the short latent period, or to the dosage being too low to evoke lesions.

There was a conspicuous weight loss in the animals exposed to pavetamine, as indicated in figure 5.1 and table 5.2. Weight loss following the administration of pavetamine was also reported by Hay, Schultz and Schutte (2008) who studied the cardiotoxic effects of pavetamine in rats. In the current trial the rats became anorexic within two to three days after exposure to pavetamine and regained weight within a few days (around day 7) after initial exposure. However, they kept on losing weight after the second exposure and this trend continued until termination of the experiment. Schultz *et al.* (2001) reported that pavetamine was an inhibitor of protein synthesis, particularly of myocardial contractile proteins. Apart from the weight loss associated with a reduced intake of food, the chronic effect of pavetamine on protein synthesis most probably contributed to the weight loss and ill thrift noticed clinically in the experimental animals.

Fourie *et al.* (1995) proved that gousiekte can be induced in ruminants by the intravenous administration of pavetamine. At the time it was not known that pavetamine was cardiotoxic to rats. Since then it has been demonstrated that

both *P. harborii* extracts and pavetamine cause heart failure in rats (Hay *et al.* 2001*;* Hay, Schultz & Schutte 2008). Pavetamine substantially reduced the systolic component of the cardiodynamic function of the rats but did not affect the diastolic component (Hay, Schultz & Schutte 2008). The cardiac failure was attributed to both the effect of pavetamine on the myocardial contractile proteins and diminished sensitivity to activating calcium ions in the myofibre contractile system. This has been confirmed in cases where myocardial damage was inflicted by means other than pavetamine (Pieske 1998).

No macro- or light-microscopical lesions associated with congestive heart failure, for example generalised congestion, lung oedema, accumulation of excessive fluid in body cavities and anasarca, were present in the rats exposed to pavetamine in the current study. The absence of these changes was also reported by Hay *et al.* (2008) in rats exposed twice to pavetamine at a dosage rate of 4 mg/kg and 3 mg/kg respectively, despite a significant reduction in systolic function. A possible explanation is that the latent period in the current study, i.e. the time from exposure of the rats to pavetamine until death, was too short for congestive heart failure to develop since compensatory mechanisms were adequate to enable the damaged heart to meet the body's circulatory demands. The absence of lesions associated with congestive heart failure was also reported in sheep that died naturally of gousiekte by Theiler, Du Toit and Mitchell (1923) and was noted in experimentally induced cases of the disease in sheep with "atypical lesions", as reported in chapter 3.

Imaging analysis was used in this study as a tool to study the effect of pavetamine on myofibres in the subepicardial and subendocardial regions of the heart. Considering the distribution of the light-microscopical myocardial lesions in ruminants with a short latent period (as outlined in chapter 3) and the rats euthanased on day 42 in this study, there appears to be no proof that pavetamine selectively affect myofibres in certain predilection sites. However, in ruminants with medium to long latent periods there is a predilection for lesions in the subendocardial region of particularly the apex and left ventricular wall (refer to chapter 3, table 3.3) (Theiler, Du Toit & Mitchell 1923; Newsholme & Coetzer 1984).

Various factors made the interpretation of the statistical analysis in rats problematic, including the striking difference in fibre diameter throughout the myocardial wall in the control group and the dramatic weight loss in rats exposed to pavetamine. Owing to these variables it was decided to establish if there was a statistical significant difference between the epicardial and endocardial measurements in control animals. If this were the case every animal could serve as its own control when comparing subendocardial and subepicardial fibres. The results showed that there was a statistically significant difference (P = <0,05) for each of the three measurements in the control animals (tables 5.3, 5.4), which was not the case in the rats exposed to pavetamine and euthanased on day 42 (tables 5.5, 5.6). This was interpreted as evidence of atrophy.

A possible explanation for atrophy of the subendocardial fibres is that this region is more severely affected than the subepicardial region as a result of ischaemia following cardiac failure. In humans, subendocardial myofibres demonstrate greater potential for ischaemic damage as a result of congestive heart failure than do subepicardial myofibres (Unverferth 1985). Furthermore, in dogs, subendocardial tissue has a 20 % greater oxygen consumption per unit weight than subepicardial tissue (Weiss *et al.* 1978). In rats, pavetamine-induced heart failure, although not clinically evidenced as congestive heart failure, results in a significantly reduced systolic function (Hay, Schultz & Schutte 2008). This may result in ischaemic damage to particularly the subendocardial tissue owing to an increased ventricular end diastolic pressure that will give rise to an increased extravascular pressure in the subendocardial interstitial tissue. This aspect of the pathogenesis of the myocardial lesions is discussed in more detail in chapter 6.

The current and previous studies confirmed the cardiotoxic effect of pavetamine in rats (Schultz *et al.* 2001; Hay, Schultz & Schutte 2008). The myocardial lesions in rats differ substantially, from the typical lesions in sheep with medium to long latent periods (as outlined in chapters 3 and 4) in that sheep show subendocardial hypertrophy, necrosis, replacement fibrosis, atrophy and round

cell infiltrates of varying intensity, especially in the subendocardial region. The nature of the myocardial lesions in the experimental rats in the current study largely resembles the lesions reported in sheep with “atypical lesions”, namely multifocal necrosis and fibre atrophy (refer to chapters 3 and 4). The subendocardial hypertrophy considered a hallmark of gousiekte in ruminants was not observed in the rats exposed to pavetamine. It is likely that rats are more susceptible to acute pavetamine poisoning than ruminants, and that if a less intense dosage regimen were applied, “typical “ gousiekte would result.